

ADA123101

Methods in Enzymology

Volume 78

Interferons

Part A

EDITED BY

Sidney Pestka

ROCHE INSTITUTE OF MOLECULAR BIOLOGY
NUTLEY, NEW JERSEY

1981



ACADEMIC PRESS

A Subsidiary of Harcourt Brace Jovanovich, Publishers

New York London Toronto Sydney San Francisco

Supersedes AD-A083563

DTIC
ELECTE
JAN 7 1983
S D

DTIC FILE COPY

062

NOTICE: THIS MATERIAL CONTAINS INFORMATION OF A CONFIDENTIAL NATURE

capodilator, pH 7.0. Under these conditions the following T_m 's are obtained for the indicated duplexes: poly(I) poly(C), 63°; poly(I) poly(br²C), 87°; poly(c¹I) poly(C), 47°; poly(c²I) poly(br²C), 83°.

[48] Staphylococcal Enterotoxin A (SEA)

By LEONARD SPERO and JOSEPH F. METZGER

The staphylococcal enterotoxins, a group of water-soluble exoproteins elaborated by certain strains of *Staphylococcus aureus*, produce an acute gastroenteritis in man and a small number of other mammalian species.¹ There are five well-defined types, A, B, C, D, and E, originally identified on the basis of serologic individuality. Serologic cross-reaction has, however, been found between types A and E.² Two cross-reacting determinants have been demonstrated in types B and C,³ and indeed some antisera show cross-immunoprecipitation between these two types.⁴

The mode of action of these enterotoxins is unknown, but it does not appear that the adenylyl cyclase-adenosine monophosphate system is involved.⁵ In addition to their emetic activity, the staphylococcal enterotoxins have also been demonstrated to be polyclonal mitogens for mouse and human splenic lymphocytes.⁶ The stimulation is essentially limited to T cells⁷; types A, B, and C are equipotent.⁸ Johnson and co-workers have shown that enterotoxin A (SEA) stimulates the production of interferon of the immune type in both splenic⁹ and peripheral¹⁰ lymphocytes. The conditions for this production of human and mouse interferon are described in this volume [75] and [77].

Assay Method

Serologic Activity. Many immunodiffusion procedures have been successfully employed, but we find the radial diffusion method of Mancini *et*

¹ E. P. Casman, M. S. Bergdoll, and J. Robinson, *J. Bacteriol.* 85, 715 (1963).

² M. S. Bergdoll, C. R. Borja, R. N. Robbins, and K. F. Weiss, *Infect. Immun.* 4, 593 (1971).

³ L. Spero and B. A. Morlock, *J. Immunol.* 122, 1285 (1979).

⁴ A. C. M. Lee, R. N. Robbins, R. F. Reiser, and M. S. Bergdoll, *Infect. Immun.* 27, 431 (1980).

⁵ F. R. DeRubertis, T. V. Zenser, W. H. Adler, and T. Hudson, *J. Immunol.* 113, 151 (1974).

⁶ D. L. Peavy, W. H. Adler, and R. T. Smith, *J. Immunol.* 105, 1453 (1970).

⁷ M. Greaves, G. Janossy, and M. Doenhoff, *J. Exp. Med.* 140, 1 (1974).

⁸ J. R. Warren, D. L. Leatherman, and J. F. Metzger, *J. Immunol.* 115, 49 (1975).

Accession		
NTIS GRA&		
DTIC TAB		
Unannounced		
Justification		
By		
Distribution		
Availability Codes		
Dist	Avail	nd/or
A	2	al

*al.*¹¹ to be particularly convenient and simple. Hyperimmune goat anti-SEA is incorporated into agar [1 ml in 50 ml of 1% agarose (Bio-Rad) dissolved in 0.2 M borate buffer at pH 8.3]. Five microliters of a series of twofold dilutions of toxin are introduced into 2-mm wells, and diffusion is allowed to proceed to equilibrium, 24–48 hr at room temperature in a moist chamber. The diameter of the ring of immunoprecipitate is measured, and the concentration estimated from a standard curve obtained with purified toxin.

Emetic Activity. The enterotoxin in phosphate-buffered saline is injected intravenously into the saphenous vein of rhesus monkeys (*Macaca mulatta*; weight about 3 kg). The administered dose should not exceed 1 µg/kg of body weight, as lethality is sometimes encountered at very high levels. The animals are observed continuously for 5 hr after administration for emesis and/or diarrhea. Threefold dilutions are tested; satisfactory assays have been obtained with four animals per dilution.

Handling Procedure

Enterotoxin A is extremely toxic to man. It should be handled with great caution. The toxin is preserved as a lyophilized powder, which should never be weighed out. It should be reconstituted with the appropriate solvent through the septum sealing the vial. Solutions of the toxin must *never* be pipetted by mouth. Contaminated glassware should be autoclaved for 15 min at 120°. Spectrophotometer cuvettes may be cleaned in strong acid.

Purification

Two methods of purification of SEA have been published.^{12,13} We describe here the modification of the method of Schantz *et al.*¹³ currently employed in our laboratory.

Fermentation. A single high-producing colony of mutant strain 13N-2909 of *S. aureus*¹⁴ was selected from antibody-agar plates, propagated on agar, and lyophilized. The lyophilized tubes have been stored at room

¹¹ L. C. Osborne, J. A. Georgiades, and H. M. Johnson, *Infect. Immun.* 23, 80 (1979).

¹² M. P. Langford, J. A. Georgiades, G. J. Stanton, F. Dianzani, and H. M. Johnson, *Infect. Immun.* 26, 36 (1979).

¹³ G. Mancini, A. O. Carbonara, and J. F. Heremans, *Immunochemistry* 2, 235 (1965).

¹⁴ F. S. Chu, K. Thadhani, E. J. Schantz, and M. S. Bergdoll, *Biochemistry* 5, 3281 (1966).

¹⁵ E. J. Schantz, W. G. Roessler, M. J. Woodburn, J. M. Lynch, H. M. Jacoby, S. J. Silberman, J. C. Gorman, and L. Spero, *Biochemistry* 11, 360 (1972).

¹⁶ M. E. Friedman and M. B. Howard, *J. Bacteriol.* 106, 289 (1971).

temperature; the organism has been stable for over 5 years. A single lyophilized tube is used for each fermentation. The culture is passed three times in shake flasks to obtain 200 ml of inoculum (the dried organisms in 100 ml of medium, 2 ml of this into 100 ml of medium, and finally 2 ml into each of two flasks containing 100 ml). Each growth period is 18–24 hr at 37°.

The medium utilized consists of 4% N-Z amine type NAK (Sheffield), 1% yeast extract (Difco), and 0.2% dextrose. For the final fermentation the sugar is sterilized by filtration and added after the remainder of the medium is autoclaved. The pH of the medium prior to inoculation is 6.7 without adjustment. A 70-liter fermentor (Fermentation Design, Inc.) containing 50 liters is used. The fermentation conditions are: 37°, 400 rpm agitation at 10 liters/min of air sparging, and antifoam (a 50% suspension of Antifoam 60, Harwick) as demanded.

After 5–7 hr, there is a slight drop in pH to 5.9–6.1, followed by a gradual rise to pH 8.0 at 24 hr. The culture is cooled, and the bacterial cells are removed by centrifugation at 20,000 rpm in a continuous-flow system (Lourdes). This supernatant assays between 90 and 100 $\mu\text{g/ml}$ by radial immunodiffusion. The solution is diluted to 200 liters with distilled water, and the pH is adjusted to 5.6 with phosphoric acid.

Chromatography on CG-50. Four-hundred and fifty grams of CG-50 (Rohm and Haas) is washed copiously to remove fines. It is then cycled between pH 11 and 3. The resin is suspended in 6 liters of 0.005 M NaH_2PO_4 and the pH is adjusted to 5.6 with 4 N NaOH. Buffer salts are removed by washing three times; the resin is added to the diluted culture supernatant. The resin is stirred in the culture for 2 hr and allowed to settle. The supernatant is pumped off the resin, which is then washed five times with water. The resin is transferred into a chromatographic column (5 × 100 cm). It is washed with 2 liters of water at a flow rate of 5 ml/min, and the toxin is eluted with 0.5 M sodium phosphate buffer at pH 6.2 in 0.5 M NaCl. The toxin, which is eluted in the second and major peak (about 3 liters) is dialyzed against water in the cold for 24 hr and then against 0.008 M sodium phosphate buffer at pH 6.0 in 0.008 M NaCl for 24 hr. This low level of buffer and salt increases the stability of the toxin. The toxin solution is clarified by centrifugation and diluted to 10 liters with water immediately prior to the next step.

Chromatography on CM-Cellulose. Carboxymethylcellulose (200 g) (Cellex CM, Bio-Rad) is washed extensively with water to remove fines. It is suspended in 6 liters of 0.01 M Na_2HPO_4 adjusted to pH 6.0 with 4 N NaOH and then washed with water. The cellulose is added to the diluted toxin-containing solution and stirred for 1 hr. It is then transferred to a chromatographic column (5 × 100 cm) and washed with one void volume of 0.008 M phosphate in 0.008 M NaCl at pH 6.0. The toxin is eluted with

TABLE I
SUMMARY OF THE PURIFICATION OF STAPHYLOCOCCAL
ENTEROTOXIN A^a

Purification step	Volume (ml)	Toxin (mg/ml)	Recovery (%)	Purity (%)
Centrifuged culture	50,000	0.09-0.10	—	—
CG-50	3,000	0.25-0.35	15-20	40-60
CM-Cellulose	300-450	0.90-1.4	50-70	80-90
Hydroxyapatite	500-600	0.75-1.2	90-95	>99

^a These values represent the ranges obtained from many runs over a period of several years. The recovery range per step is given in the table.

a linear gradient of 0.01 M phosphate buffer at pH 6.0 and 0.05 M phosphate at pH 6.8 (5 liters of each buffer) at a flow rate of 5 ml/min. The pooled fractions containing the toxin are adjusted to pH 5.7 and centrifuged to remove suspended material.

Chromatography on Hydroxyapatite. Hydroxyapatite (200 g; BioGel, HTP, Bio-Rad) is equilibrated in 0.03 M sodium phosphate buffer at pH 5.7. A column (5 × 50 cm) is poured, and one void volume of the same buffer is run through at a rate of 2-3 ml/min. The toxin solution is applied to the column at a rate of 2-3 ml/min. The toxin is eluted with a linear gradient of 0.2 to 0.4 M phosphate buffer at pH 5.7 (3 liters of each buffer) at a flow rate of 2-3 ml/min. An overall yield of about 0.5 g is obtained. The purification process is summarized in Table I.

Packaging of the Purified Enterotoxin

The peak from the hydroxyapatite is dialyzed against 0.001 M phosphate buffer at pH 6.8 and lyophilized. The toxin is reconstituted in water to give a concentration of about 10 mg/ml. It is dialyzed against 0.01 M phosphate buffer at pH 6.8 and centrifuged to remove any insoluble material. Suitable aliquots are lyophilized in vaccine bottles and sealed under vacuum. The dried toxin is stored at -20°.

Chemical, Biological, and Physical Properties

The purified protein gives only a single line of immune precipitate in Ouchterlony and Oakley double-diffusion tests against an antiserum prepared from crude culture. It contains no α - or β -hemolysin, and tests for dermonecrotic substances are negative. The intravenous median effective dose (ED₅₀) in rhesus monkeys was determined as 0.03 μ g/kg of body weight.¹³ By the oral route the ED₅₀ is 1 μ g/kg of body weight in mon-

TABLE II
SOME PHYSICOCHEMICAL PROPERTIES OF PURIFIED
STAPHYLOCOCCAL ENTEROTOXIN A

Property	Description
Appearance (lyophilized)	White fluffy powder
Type of protein	Simple (contains amino acids only)
Nitrogen content (%)	16.2
Sedimentation coefficient, $s_{20,w}$ (S)	3.03
Diffusion coefficient, $\text{cm}^2 \text{sec}^{-1}$ (D)	9.8×10^{-7}
Partial specific volume, $\text{cm}^3 \text{g}^{-1}$ (amino acid composition)	0.732
Molecular weight (determined by)	
Sedimentation equilibrium	27,800
Gel electrophoresis	27,500
<i>s</i> , D	28,000
Amino acid analysis	27,970
Isoelectric point (isoelectric focusing at 4°)	7.4 (major component)
Maximum absorption (nm)	277
Extinction ($E_{1\%}^{1\text{cm}}$)	14.6

keys. Staphylococcal enterotoxin A appears to have a higher specific activity than enterotoxin B or C.

Biological activities are retained after heating a solution of the toxin at 60° at pH 7.3 for as long as 16 hr. At room temperature the toxin is stable for several days in 0.05 M phosphate buffer at pH 6.8. The lyophilized toxin is stable for at least 5 years when stored at -20°. The kinetic stability of SEA is considerably less than that of SEB or SEC; e.g., SEA unfolds 50 times faster in 8 M urea than the other enterotoxins.¹⁵

Purified SEA is homogeneous in the analytical ultracentrifuge. It gives a single line in disc electrophoresis at pH 4.3 and in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Some of its chemical and physical properties are summarized in Table II.¹⁵ It will be noted that the isoelectric point obtained by isoelectric focusing is given only for its major component. In common with SEB and SEC, enterotoxin A always displays a paucidispersity in isoelectric focusing demonstrable both in polyacrylamide gels and in sucrose-stabilized ampholine pH gradient. We have shown with SEB¹⁶ that these components arise by amide hydrolysis. The rate of *in vitro* conversion is, however, too slow to account for the appearance of the four components during the comparatively short period of the bacterial fermentation; it was suggested that only the most basic

¹⁵ J. R. Warren, *J. Biol. Chem.* **252**, 6831 (1977).

¹⁶ L. Spero, J. R. Warren, and J. F. Metzger, *Biochim. Biophys. Acta* **336**, 79 (1974).

TABLE III
AMINO ACID COMPOSITION OF STAPHYLOCOCCAL ENTEROTOXIN A

Amino acid	Residues/molecule	Amino acid	Residues/molecule
Lysine	25	Alanine	8
Histidine	7	Half-cystine	2
Arginine	7	Valine	14
Aspartic acid	38	Methionine	2
Threonine	17	Isoleucine	10
Serine	10	Leucine	25
Glutamic acid	27	Tyrosine	19
Proline	4	Phenylalanine	8
Glycine	15	Tryptophan	2
		Amide groups	31
Total residues: 240			

component is synthesized by the microorganism and that this is converted enzymically to the other forms. Similar considerations would appear to be applicable to SEA.

Enterotoxin A is a simple protein composed of a single polypeptide chain containing one disulfide bridge and no free sulfhydryl groups. This basic structure is also present in enterotoxins B and C. The amino acid composition of SEA is presented in Table III.¹³ The 240 residues give a molecular weight of 27,970, which is in good agreement with the values obtained by physical measurements. The molecular weight of SEA is very close to that of SEB and SEC.

Both the amino terminal¹⁷ and carboxyl terminal¹³ residues are serine. A tentative identification of an amino terminal alanine¹⁸ and a failure to find any amino terminal residue¹³ were reported on earlier preparations. The toxin preparation described here is readily sequenced by the automated Edman procedure. Our laboratory has described the circular dichroic spectra of enterotoxin A, B, and C.¹⁹ Although there are features unique to each type, the basic folding into secondary and tertiary structure is similar for all three. The molecules appear to possess only a very low level of α -helix.

¹⁷ J.-Y. Huang, personal communication, 1979.

¹⁸ M. S. Bergdoll, in "Biochemistry of Some Foodborne Microbial Toxins" (R. I. Mates and G. N. Wogan, eds.), p. 1. MIT Press, Cambridge, Massachusetts, 1967.

¹⁹ J. L. Middlebrook, L. Spero, and P. Argos, *Biochim. Biophys. Acta* 621, 233 (1980).